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Effect of microstructure on population growth parameters of *Escherichia coli* in gelatin-dextran systems

Kathleen Boons[a,b], Estefanía Noriega [a,b], Rob Van den Broeck[b], Charlotte C. David[c], Johan Hofkens[c], Jan F. Van Impe [a,b]

[a]CPMF[2] - Flemish Cluster Predictive Microbiology in Foods – www.cpmf2.be

[b]BioTeC - Chemical and Biochemical Process Technology and Control, Department of Chemical Engineering, KU Leuven, Leuven, Belgium, [kathleen.boons, estefania.noriegafernandez, rob.vandenbroeck, jan.vanimpe]@cit.kuleuven.be

[c]Molecular Visualization and Photonics, KU Leuven, Leuven, Belgium, [charlotte.david, johan.hofkens]@chem.kuleuven.be

Correspondence to:

Prof. J. F. Van Impe

Chemical and Biochemical Process Technology and Control (BioTeC),

Department of Chemical Engineering, KU Leuven,

W. de Croylaan 46, B-3001 Leuven (Belgium)

jan.vanimpe@cit.kuleuven.be

Tel: +32-16-32.14.66

Fax: +32-16-32.29.91

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Abstract

Literature acknowledges the effect of food structure on bacterial dynamics. Most studies introduce this "structure" factor using a single gelling agent, resulting in a homogeneous environment, while in practice most food products are heterogeneous. Therefore, this study focuses on heterogeneous protein-polysaccharide mixtures, based on gelatin and dextran. These mixtures show phase separation leading to a range of heterogeneous microstructures by adjusting relative concentrations of both gelling agents. Based on confocal microscope observations, growth of *Escherichia coli* in gelatin-dextran systems was observed to occur in the dextran phase. To find a relation between microscopic and population behavior, growth experiments were performed in binary and singular gelatin-dextran systems and culture broth at 23.5°C, with and without adding 2.9% (w/v) NaCl. The Baranyi and Roberts growth model was fitted to the experimental data and parameter estimates were statistically compared. For salted binary mixtures, a decrease of the population maximum cell density was observed with increasing gelatin concentration. In this series, for one type of microstructure, i.e., a gelatin matrix phase with a disperse dextran phase, the maximum cell density decreased with decreasing percentage of dextran phase. However, this relation did not longer hold when other types of microstructure were observed. Compared to singular systems, adding a second gelling agent in the presence of NaCl had an effect on population lag phases and maximum cell densities. For unsalted media, growth parameters of singular and binary mixtures were comparable. Introducing this information into mathematical models leads to more reliable growth predictions and enhanced food safety.

Introduction

The Centers for Disease Control and Prevention (CDC) estimate that each year roughly 1 in 6 Americans (i.e., 48 million people) gets sick, 128,000 are hospitalized, and 3,000 die of foodborne diseases (1). In the EU, more than 55,000 human cases associated to food borne outbreaks were reported in 2012 (2). Predictive microbiology is one of the approaches to improve food safety. It aims at constructing mathematical models that predict microbial behavior as a function of environmental conditions. These predictions are useful tools in risk assessment, food process control and product design (3).

Microbial behavior in liquid systems has been studied thoroughly and most available predictive models are based on experimental data in broth. These models are routinely applied to predict microbial growth in structured food systems, although food structure has been acknowledged to play a key role on microbial growth (4). Food model systems that mimic the composition and structure of real foods are often used to study microbial behavior in foods under reproducible and controllable conditions. A myriad of studies on microbial behavior in structured systems have already been conducted, involving different gelling agents and target microorganisms (see Table 1).

Most studies involving food model systems only handle one gelling agent, resulting in a homogeneous growth environment. In contrast, most food products contain different phases, e.g., water, proteins, polysaccharides and fat, leading to a heterogeneous environment. Observation of bacterial growth in such heterogeneous systems is mostly limited to studies in specific food products (see Table 1). No general conclusions can be drawn about the sole effect of a heterogeneous microstructure on microbial growth as these food products contain each their own characteristic amount of nutrients, salt, preservatives etc. General studies in model systems with a

heterogeneous microstructure have already been performed (see Table 1). It has been shown that the microstructure of packed beds (22, 23) and emulsion systems influences microbial behavior (24, 20, 21). However, in these studies, heterogeneity is introduced by the addition of a second phase that does not allow bacterial growth, and in the case of the packed bed, not resembling real food products as silica particles and Sephadex microspheres are not used as food components in real food products. In summary, to accurately predict microbial behavior in food products, heterogeneous food model systems with all phases potentially supporting bacterial growth must be studied, meaning that microbial growth in these phases is physically possible. However, the components that introduce heterogeneity (e.g., gelling agents) do not necessarily have to be metabolizable.

Proteins, polysaccharides and their mixtures are widely used in food products (33) and are known to show phase separation in a certain range of conditions, e.g., at high ionic strength (34). The process of phase separation and the resulting heterogeneous microstructures have extensively been discussed in literature (e.g., 34, 35). Additionally, it is well-known that bacterial growth is supported by nutrient-enriched protein (10, 15, 12, 8) and polysaccharide based gels (9). Examples of food products containing protein-polysaccharide mixtures include puddings, whipped cream, sauces and dressings.

The general objective of this study is to investigate the effect of different heterogeneous microstructures on the microbial dynamics of *Escherichia coli* by performing growth experiments in phase separating protein-polysaccharide food model systems, i.e., gelatin-dextran (G/D) systems. The present work studies population growth dynamics of *Escherichia coli* in seven gelatin-dextran mixtures holding different microstructures obtained by using different ratios of gelatin and dextran, as created in (37). In this series of experiments, gelatin/dextran ratios equal or bigger than one were tested. To study the effect of salt, added in order to ensure phase

separation, on the phase separation of the G/D systems and on the microbial growth behavior in the G/D systems, experiments are also performed in binary systems without added salt. As the dextran phase is the preferential phase for *E. coli* growth, it is tested whether singular dextran systems can mimic the population growth behavior in the binary G/D systems. For this purpose, results in binary systems are compared with those obtained in singular systems of gelatin and dextran and, for completeness, in culture broth, with and without added salt. Growth parameters are estimated by fitting the Baranyi and Roberts growth model (38) to the experimental data. Population results are compared with confocal microscope images in order to find a relation between microscopic and population observations.

This is the first study that investigated population microbial dynamics in binary gelled systems with heterogeneous microstructures supporting microbial growth in relation with confocal microscope observations. In addition, some hypotheses for the observed phase separation and preferential growth phase are presented.

Material and methods

Microorganisms and preculture conditions

Escherichia coli JM-109 DE3 pRSETb-Venus stock culture was kindly provided by the Department of Chemistry (KU Leuven, Belgium). Inoculum was prepared by transferring a loopful of the stock culture into an Erlenmeyer containing 20 mL of brain heart infusion (BHI) (Oxoid, Basingstoke, UK) enriched with 20 µL of ampicillin (Applichem, Darmstadt, Germany). After 9 h at 37°C under static conditions (Binder KB-series incubator; Binder Inc., NY, USA), a 20 µL aliquot of the stationary phase culture was inoculated into 20 mL of fresh BHI containing 20 µL ampicillin and incubated for 15 h under the same conditions.

Gelled media: preparation, characterization and inoculation

Gelled media were prepared by mixing BHI powder (37 g/L), and if appropriate 2.9% (w/v) NaCl (Analar NORMAPUR, VWR, Belgium), with different ratios of gelatin (Gelatin from Bovine skin, type B, Sigma, USA) and dextran (Dextran from *Leuconostoc* spp. Mr ~ 500000, Sigma, Denmark) in glass tubes with screw cap. The salt is added to ensure high enough ionic strength and hence phase separation in accordance with Tromp and coworkers (36). Different ratios of gelatin and dextran were chosen in order to unravel the effect of their relative concentration on system microstructure and microbial behavior (see Table 2). After adding 15 mL of demineralized water, samples were placed in a thermostatic water bath (GR 150 S12, Grant, UK) at 70°C for 12 min. In a next step, 30 µL of a 0.01% (w/v) Rhodamine B-solution (R953, Aldrich, Germany) was added and the mixture was filter sterilized through a 0.2 µm pore size filter (Filtropur S 0.2, SARSTEDT, Germany) with the aid of a syringe (10 mL Norm-Ject, Henke Sass Wolf, Germany). 15 µL of ampicillin was added to the samples and then, the appropriate volume of a dilution of the preculture was inoculated to obtain an initial cell density of about 10^4 CFU/mL. Undiluted samples are difficult to plate due to the consistency of the gelled media, so inoculum level was chosen in such a way that the first decimal dilution of the first sample could be detected via plate counting.

Water activity and pH of each mixture were determined with the aid of an aw-Kryometer (AWK-40, NAGY, Germany) set on ‘liquid’-modus and a pH meter (DocuMeter, Sartorius, Germany), respectively. Measurements were performed on media, without bacterial cells.

Experimental setup for growth experiments

5 mL sterile glass screw-cap tubes were filled with 1 mL of inoculated medium and then, phase separation was induced by incubating the samples at room temperature for 20 min. After 6 min at 4°C to solidify the gels and again, 4 min at room temperature to warm them up, samples were placed in a temperature controlled water bath at 23.5°C. A water bath was used in order to obtain fast heat transfer. Within 5 min, the temperature in the tubes reaches the temperature in the water bath. The temperature selected for growth experiments is a trade-off between the temperature at which the gelatin-dextran mixture is stable and the temperature that can be kept fixed in a water bath without cooling.

At regular time steps, one tube of each mixture was removed from the water bath at 23.5°C and placed in another water bath at 37°C, in order to melt the structured medium. After preparing the appropriate serial decimal dilutions in BHI, samples were plated onto BHI supplemented with 1.4% (w/v) agar (Agar technical No. 3, Oxoid, Basingstoke, UK). Plates were incubated for at least 18 h at 37°C before viable cell counting. At least four independent experiments were conducted for each mixture.

Estimation of growth parameters

The growth model of Baranyi and Roberts (38) was fitted to the growth curves. Model parameters were estimated from the set of experimental data corresponding to each mixture via the minimization of the sum of squared errors (SSE), using the lsqnonlin routine with the Levenberg-Marquardt optimization algorithm of the Optimization Toolbox of Matlab version R2010b (The Mathworks, Inc., Natick, MA, USA). Standard errors of parameter estimates were calculated from the Jacobian matrix. It has to be pointed out that a global estimation procedure was standardized for each mixture to consider deviations in test reproducibility.

157 **Statistical analysis**

158 Analysis of variance (ANOVA) test was performed to determine whether there are any
159 significant differences amongst means of parameter estimates, at a 95.0% confidence level (α =
160 0.05). If ANOVA indicated significant differences between the parameters for the different
161 mixtures, a Fisher´s Least Significant Difference (LSD) test was used to identify which means
162 were significantly different. Standardized skewness and standardized kurtosis were used to assess
163 if data sets came from normal distributions. These analyses were performed using Statgraphics
164 Centurion XVI.I® Package (Statistical Graphics, Washington, USA). Test statistics were
165 regarded as significant when P was ≤ 0.05. Analyses were performed for experiments with and
166 without added salt separately.

167

168 **Microscopy: sample preparation and image analysis**

169 Preparation of the mixtures for the confocal microscope samples was performed in the same way
170 as for the growth experiments. After inoculation, well chambers (chambered borosilicate
171 coverglass system, Nunc Lab-Tek ,USA) were filled with 300 µL of the mixture and incubated at
172 room temperature. As described in (37), images were taken with a commercial laser scanning
173 microscope (FV 100, Olympus, 60X magnification). An image analysis procedure was written
174 using the algorithms embedded in the Matlab Image Processing Toolbox (The Mathworks, Inc.,
175 Natick, MA, USA). At least 5 images per mixture were analyzed. First, microscope RGB images
176 were converted to grayscale images. Next, contrast was enhanced by applying adaptive histogram
177 equalization. Finally, the contrast enhanced grayscale images were segmented with pixel
178 thresholding. These segmented images allow quantifying size and shape characteristics of the

179 microstructures in the samples. In this paper, the percentage of dextran phase in the images is
180 reported as surface fraction in the analyzed images.

181 # Results

182 ## Intrinsic properties of G/D systems: pH and water activity

183 In Figure 1, water activity and pH of the different studied systems are plotted as a function of
184 medium composition. These population measurements are performed in mixtures without the
185 addition of bacteria. For the mixtures supplemented with salt, water activity values range from
186 0.9687 (4G/1D) to 0.9799 (1D). Values for mixtures with no added salt vary between 0.9914
187 (4G) and 0.9970 (1D). pH values fluctuate between 6.17 (4G) and 7.29 (1D), for the salted
188 mixtures, and from 6.30 (4G/1D) to 7.08 (1G/1D), for the non-salted mixtures. The variation in
189 pH and water activity is induced by changing the composition of the mixture, i.e., adding dextran,
190 gelatin and salt. The addition of salt or gelatin causes a decrease in water activity and pH,
191 whereas the effect of dextran is limited.

192 ## Microscopic characterization of G/D systems

193 The confocal microscope images taken in salted and unsalted mixtures are depicted in Figure 2.
194 For all systems, phase separation between gelatin and dextran is observed. For the same ratio of
195 added gelling agents, microstructures are similar for salted and unsalted mixtures. Only for the
196 1G/1D and 2G/1D systems, a bi-continuous phase is observed for the unsalted mixtures whereas
197 the microstructure in the salted systems consists out of a disperse phase in a matrix phase. For
198 salted as well as for unsalted mixtures, phase inversion is observed when changing the ratio of
199 gelling agents from 4G/1D to 2G/2D. As was previously observed in the salted systems (37), also

in the unsalted systems *E. coli* growth preferable occurs in the dextran phase, regardless of the microstructure.

Population growth dynamics in G/D systems with added salt

In Figure 3a, *E. coli* growth curves obtained at the population level in singular and binary gelatin-dextran mixtures and liquid BHI with added salt are shown. For all conditions, sigmoidal curves including the typical microbial growth phases are observed. **Population growth kinetics: lag phase duration and maximum growth rate** From Figure 3a it is clear that the population lag and exponential growth phase in binary G/D systems are similar for all mixtures. However, growth curves obtained in singular dextran systems and also in BHI broth show a delay in population lag phase, as compared to binary systems. This is contra-intuitive, i.e., the dextran phase has been shown to be the preferential site for *E. coli* in gelatin-dextran systems, hence it was expected that population growth in binary systems would be comparable to that observed in singular dextran systems. Results of the parameter estimation confirm the previously mentioned observations (Figure 4a-4c, including results from ANOVA and LSD tests). For the salted G/D mixtures, population lag phase durations do not vary significantly among binary mixtures, excluding the 1G/1D-system, which has a longer population lag phase (Figure 4a). However, a significantly higher value is observed for the population lag phase duration in the liquid system and the singular dextran systems, with a negligible difference between both dextran systems. The population lag phase duration in the singular gelatin system is within the range of the values observed in the binary systems but significantly lower than the values obtained for the singular dextran systems. Looking to the different parameter values for the population maximum specific growth rate, an increasing trend is observed with increasing gelatin concentration.

Population maximum growth capacity Figure 4c indicates that increasing the gelatin concentration for a fixed dextran concentration results in a slight decrease in the population maximum cell density. A similar but less pronounced observation holds for an increase in dextran concentration, keeping the gelatin concentration constant. The population maximum cell density in the singular gelatin system is lower than the other reported concentrations, whereas values for the BHI broth and singular dextran systems are within the range of those obtained for the binary mixtures. Again, the difference between both singular dextran systems is negligible.

Population growth dynamics in G/D systems with no added salt

As for the salted systems, for all systems with no added salt, sigmoidal growth curves are observed (Figure 3b). In contrast to what is observed for the salted systems, Figure 3b illustrates that *E. coli* growth curves in systems with no added salt coincide for binary and singular systems, as well as for liquid BHI.

Population growth kinetics: lag phase duration and maximum growth rate As expected, a reduction of the salt content to 0.5%, i.e., the percentage of salt present in BHI, leads to a decrease in population lag time and an increase in population maximum growth rate and maximum cell density. Parameter estimation (Figure 4d and 4f, including results from ANOVA and LSD tests) indicates that variations in population lag time and population maximum growth rate obtained for results in unsalted mixtures are rather limited.

Population maximum growth capacity An increase in population maximum cell density is observed when comparing parameter estimates for systems without and with added salt. For the population maximum cell density in mixtures with no added salt, a similar trend as for the salted media can be observed when increasing the gelatin concentration for a fixed dextran

concentration, i.e., the population maximum cell density decreases. However, this trend is more pronounced for the salted mixtures.

Discussion

Microscopic characterization of G/D systems

In a previous work (37), phase separation in G/D systems, supplemented with BHI and 2.9% salt, was observed. Changing the relative and absolute amount of the gelling agents led to a change in microstructure characteristics. More specifically, when increasing the amount of gelatin for the 1D systems, the diameter of the dextran spheres increased (Figure 2a). When adding bacteria, it was found that the dextran phase was the preferential site for *E. coli* growth, regardless of the microstructure. However, the underlying mechanisms for these observations were not unraveled and further analysis is encouraged. In this work, the observed phase separation as well as the preferential phase-behavior are discussed in detail and supported with relevant literature.

Phase separating G/D systems First of all, the mechanisms behind phase separation in protein-polysaccharide mixtures, like G/D systems, are discussed. It is well-known that, under specific conditions, mixtures of proteins and polysaccharides show thermodynamic incompatibility, forming Aqueous Two-Phase Systems (ATPS). This incompatibility between proteins and neutral polysaccharides can be reached at high ionic strengths or at pH values different from the isoelectric point of the protein (pI). Also, the difference in hydrophilicity between proteins and polysaccharides, the so-called $\Delta\chi$-effect, is of great importance for phase equilibrium in protein-polysaccharide-water systems (34). For the mixture gelatin-dextran, Grinberg and Tolstoguzov (34) reported that incompatible conditions for the protein-polysaccharide mixture are fulfilled

when i) the ionic strength is lower than 0.15 and the pH is equal to the pI or ii) the ionic strength is higher than 0.15 and there is an inequality between pH and pI. With high ionic strength (> 0.5 M) and pH values for all salted mixtures (Figure 1b) that exceed the pI of gelatin (4.7-5.2) (39), phase separation is expected to happen in the salted mixtures, as already described in literature (36, 35). In this work, it was observed that when adding no extra salt, phase separation still occurs. As the pH of these mixtures exceeds the pI of gelatin and the added BHI causes an ionic strength of 0.38 M, conditions for phase separation are fulfilled. In the non-salted systems, phase separation is observed for all systems, including the 1G/1D systems, although for this system phase separation was not evident when salt was added.

Distribution of *E. coli* cells in phase-separating G/D systems The distribution of bacterial cells in different ATPS is already described in literature. However, to the best of the authors' knowledge, no studies focus on the growth behavior of microorganisms in ATPS with different compositions and, hence, different microstructures. Also, most studies are performed in systems not resembling food products. *Clostridium tetani*, *Lactobacillus rhamnosus*, *E. coli* W3110, ML308 and MG1655 were shown to have a preference for the dextran phase in polyethylene glycol-dextran systems (40, 42, 41, 43). Also, in polyvinylpyrrolidone-dextran systems, *Lactobacillus rhamnosus* and *Enterococcus faecum* M74 were found preferentially in the dextran phase (42, 44). In alginate-sodium caseinate mixtures, bacteria were not located in the polysaccharide phase as observed in the present study but *Lactococcus lactis* LAB3 preferred the protein phase (45).

In the present study, *E. coli* cells were located in the dextran phase of the G/D system. In what follows, some hypotheses for this behavior are discussed. In the G/D mixtures studied here, the pI of gelatin is exceeded, which makes the protein become negatively charged. Harden and

coworkers (46) reported that *E. coli* cells have a negative cell surface charge in a suspending solution of 0.01M K_2HPO_4 and 0.01M KH_2PO_4 (pH 7.0). Schwarz-Linek and coworkers (47) performed electrophoretic mobility measurements and confirmed that *E. coli* carried a net negative charge in modified phosphate buffer. Also Dickson and coworkers (48) measured a negative charge on the *E. coli* cell surface when suspended in a phosphate buffer and its effect on bacterial attachment to meat surfaces. Because of this negative charge, electrostatic repulsion is likely to be one of the reasons why the cells do not appear in the proximity of the negatively charged gelatin molecules but prefer the neutral dextran phase. However, it must be mentioned that *E. coli* can be found in the gelatin phase in singular gelatin systems.

In literature, it is stated that surfaces of *E. coli* cells are relatively hydrophilic compared to proteins, probably because of the hydrophilic head groups of the phospholipids, which are the main components of the cell membrane (49, 50). As dextran is known to be a hydrophilic molecule (51), it is likely that the hydrophilicity of both dextran molecules and *E. coli* cells makes this phase the preferential site for *E. coli* growth. On the other hand, gelatin is a molecule containing hydrophilic as well as hydrophobic groups (52).

In conclusion, the combination of both electronegativity and hydrophilicity favors the distribution of *E. coli* in the dextran phase over the gelatin phase. As such, both mechanisms contribute to the observed preferential phase behavior.

Population growth dynamics in G/D systems with added salt

Estimated growth parameters in salted binary and singular mixtures are compared as a function of the mixture composition in order to unravel the effect of system microstructure on population

312 bacterial dynamics. In this work the relation between observed microstructure and population
313 growth parameters is discussed.
314
315 **Population growth kinetics: lag phase duration** When comparing the population lag phase
316 duration of the different mixtures, significantly different values can be observed for the 1G/1D
317 binary mixture, the singular dextran systems and the liquid system. The absence/lower amount of
318 gelatin in these systems is a coherent explanation for this increase in population lag phase. Boons
319 and coworkers (8) studied growth behavior of *E. coli* and *Salmonella* Typhimurium in gelatin,
320 xanthan gum and gelatin-xanthan gum systems and in BHI broth at 23.5°C, at salt concentrations
321 ranging from 0.0 to 5.0% (w/v). They found that the addition of gelatin to BHI broth caused a
322 decrease in population lag phase at salt concentrations of 3.0, 4.0 and 5.0% (w/v). In this sense,
323 sodium chloride has reported to be bound to gelatin (52, 54), which can cause a lower apparent
324 salt concentration. As the population lag phase duration is influenced by cell prehistory and
325 environmental growth conditions, a lower apparent salt concentration in the growth medium, due
326 to a higher gelatin concentration, may lead to shorter population lag phases, given a non-salt
327 enriched preculture medium. As 2.9% (w/v) NaCl is added to all systems, differences in
328 population lag phase duration can be expected, depending on the gelatin concentration. In the
329 singular dextran systems and BHI broth, no gelatin is present in the media, which can explain the
330 longer population lag phase. For the 1G/1D system, the gelatin concentration may not be high
331 enough to cause the full effect on salt concentration observed in the other binary mixtures,
332 although it affects significantly population lag times, as compared with singular dextran systems
333 and BHI broth. This hypothesis is also supported by the experimental results in systems with no
334 added salt, where population lag times are comparable, independently of mixture composition.

Population growth kinetics: maximum growth rate Statistical analysis of population maximum specific growth rate estimates for binary mixtures (Figure 4b) results in significantly different values. Increasing the gelatin concentration for a fixed dextran concentration, from 2.5 (for the 1D systems) or 5 (for the 2D systems) to 10% (w/v) leads to an increase in maximum specific growth rate. However, the largest difference between two values is only 0.039 h^{-1}, taking into account the minimum (0.3574± 0.0077 h^{-1} for the 2G/2D system) and maximum value (0.3962± 0.0093 h^{-1} for the 4G/2D system). Comparing growth curves of *S. Typhimurium* obtained by Theys and coworkers (12) in 1.0 and 5.0% (w/v) gelatin performed at conditions comparable to those used in the current study, an effect of the gelatin concentration on the population maximum growth rate is hardly noticed. Antwi and coworkers (15) observed that for *Listeria innocua* within the range between 5.0 and 20.0% (w/v) gelatin, the population maximum growth rate remained approximately constant and was not affected by the increase in gelatin concentration. The small difference between minimum and maximum value, combined with the information obtained from literature cannot lead us to a definitive conclusion. The population maximum growth rate of the singular gelatin system is in line with the values observed for the binary systems. However, population maximum growth rates in the singular dextran systems and especially in BHI broth turn out to be slightly lower. Possibly, the absence of gelatin and hence, the possible higher apparent salt concentration, causes a decrease in the population maximum growth rate. However, in the study of Boons and coworkers (8), discussed before, no effect of the addition of gelatin to BHI broth was observed on the population maximum growth rate.

Population maximum growth capacity It is observed that the trend in maximum cell density can be related to two factors.

Firstly, for the binary mixtures, increasing the gelatin concentration for a fixed dextran concentration, leads to a decrease in the maximum cell density. In addition, the lowest population maximum cell density is observed for growth experiments in singular gelatin systems. This is an indication that the concentration of gelatin influences to some extent the population maximum cell density. It is hypothesized that this effect may be twofold: i) by adding more gelatin, the pH and water activity are lowered. This leads to less optimal growth conditions which may affect the population maximum cell density, ii) the rigidity of the systems increases when adding more gelatin. Theys and coworkers (12) performed rheological oscillatory measurements and concluded that gels became more firm and rigid when increasing the gelatin concentration from 1.0 to 5.0% (w/v). In this more rigid structure, diffusion of nutrients towards and diffusion of metabolites away from the colony would be slowed down, which limits colony growth.

Secondly, changing the gelatin (and dextran) concentration also causes a change in microstructure. Next to the change in composition, also the resulting change in microstructure can have an effect on the population maximum cell density. Since the dextran phase is the preferential phase for bacterial growth, it is expected that the more dextran phase is present, i.e., the more space is available for bacterial growth, the higher the population maximum cell density will be. Results of image analysis suggest that there is indeed a relation between the fraction of dextran phase in the mixture and the population maximum cell density, i.e., the maximum cell density increases with increasing available dextran phase. However, this trend is only observed for mixtures exhibiting a microstructure with a dextran phase dispersed in a gelatin matrix , i.e., the 1D-binary systems show a decrease in population maximum cell density with decreasing percentages of dextran phase: 2G/1D (24.9% ± 2.5% dextran phase) - 3G/1D (21.2% ± 0.5% dextran phase) - 4G/1D (19.5% ± 1.0% dextran phase). When comparing with mixtures with a

different type of microstructure, i.e. gelatin phase dispersed in a dextran matrix or a combination of both, the relation between the population maximum cell density and the percentage of dextran phase does not longer hold. For example, the dextran fraction in the 2G/2D mixture is higher (60.8% ± 4.5%) than the dextran fraction in the 2G/1D mixture but the population maximum cell density is lower. Also the population maximum cell density in the singular dextran systems, fully consisting of dextran phase, is not higher than the values observed for the binary systems, which contain a lower fraction of dextran phase.

It must be noted that going from the 1G/1D to the 4G/1D mixture, the observed dextran spheres get bigger (Figure 2) but the population maximum cell density does not increase as expected. It seems that for the 1D-binary systems the overall percentage of dextran phase instead of the local size of a dextran sphere is the factor related to the maximum cell density. However, it should be taken into account that i) the microstructure of G/D systems depends on the concentration of the gelling agent. To uncouple these factors, future research has to focus on the creation of different microstructures, obtained with the same concentration of gelling agents, e.g., by shearing the system (55). ii) Care has to be taken when relating the population and microscopic level. The maximum cell density is a population parameter. It is an average, global value for the population present in the system, while (confocal) microscope images focus on one cell/colony (e.g., 56). By using the average value, information at the colony level might get lost, whereas confocal microscope images fail to provide an overview of the whole population.

Population growth behavior in G/D systems with no added salt

Population growth kinetics: lag phase duration and maximum growth rate. As widely accepted in literature (10, 12, 57, 8), it is also observed in this study that leaving out the extra

amount of salt has a positive effect on bacterial dynamics in G/D systems. Parameter values for the population lag time in systems with no added salt are lower and values for population maximum growth rates are higher than those obtained in the corresponding systems with extra salt. In addition, values for the population lag time are similar for all systems, whereas in the salted systems some differences in population lag times were observed. As well as for the salted systems, differences in the population maximum growth rates for the systems without added salt are considered too small to draw conclusions from.

Population growth capacity. Decreasing the salt concentration leads to an increase in population maximum cell densities. Also for these higher values, the trend of the population maximum cell density as a function of the mixture composition is similar to that observed in the salted systems, however less pronounced.

Comparing population growth capacity with observed microscopic images, it can be seen that in the 1D-binairy systems a change in type of microstructure is observed when increasing the gelatin concentration, i.e., from bi-continuous to a dextran phase dispersed in gelatin matrix, leaving no more than two mixtures with the same type of microstructure. Also for the 2D-binary systems, different microstructures are observed. As such, no conclusions on the effect of microstructure on the population maximum cell density can be drawn for the systems without added salt.

Conclusion

As a general conclusion of the performed growth experiments, we can state that to estimate the effect of a biphasic microstructure on *E. coli* growth at a macro scale, it is not sufficient to perform experiments in the preferential phase, especially when a stress factor is present, i.e., salt.

426 This is in contrast to what sometimes is mentioned in literature (e.g., 58). In the presence of salt,
427 adding a second gelling agent, influences the population lag time duration and, to a limited
428 extent, the maximum growth rate. However, for media without added salt, growth parameters of
429 singular and binary gelatin-dextran mixtures are comparable.
430 The presence of a second gelling agent, introducing a heterogeneous microstructure, also has an
431 effect on the population maximum cell density. There is a relation between the percentage of
432 dextran phase in the mixture and the maximum cell density for the salted 1D-binary systems, i.e.,
433 the mixtures with a microstructure exhibiting a dispersed dextran phase in a gelatin matrix.
434 However, this relation does not longer hold when comparing percentages of dextran phase with
435 population maximum cell densities in other types of microstructure. In the future, research is
436 needed to investigate whether there is a relation between the different types of microstructure,
437 i.e., whether there is a general parameter (e.g., rigidity of the microscopic growth environment,
438 particle size distribution,…) that can characterize the different types of microstructure in relation
439 with population growth parameters.
440 This study emphasizes that the addition of a combination of gelling agents to food products,
441 leading to a phase separated system, cannot be neglected when making predictions on population
442 bacterial growth, certainly not when additional stress factors are present.
443

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456

References

1. CDC, Centers for Disease Control and Prevention. 2011. CDC Estimates of foodborne illness in the United States. Available online: www.cdc.gov/foodborneburden/ (accessed 28.01.2014).
2. EFSA. 2014. Scientific report of EFSA and ECDC: The European Union Summary Report on Trends and Sources of Zoonoses, Zoonotic Agents and Food-borne Outbreaks in 2012. EFSA Journal.**12(2):**3547.
3. McMeekin TA, Olley J, Ratkowsky DA, Ross T. 2002. Predictive microbiology: towards the interface and beyond. Int. J. Food Microbiol. **73(2-3)**:395-407.
4. Wilson PDG, Brocklehurst TF, Arino S, Thuault D, Jakobsen M, Lange M, Farkas J, Wimpenny JWT, Van Impe JF. 2002. Modelling microbial growth in structured foods: towards a unified approach. Int. J. Food Microbiol. **73**:275-289.
5. Mitchell AJ, Wimpenny JWT. 1997. The effect of agar concentration on the growth and morphology of submerged colonies of motile and non-motile bacteria. J. Appl. Bacteriol. **83**:76-84.
6. Skandamis PN, Brocklehurst TF, Panagou EZ, Nychas G-JE. 2007. Image analysis as a mean to model growth of *Escherichia coli* O157:H7 in gel cassettes. J. Appl. Microbiol. **103**:937-947.
7. Noriega E, Laca A, Díaz M. 2010. Decisive role of structure in food microbial colonization and implications for predictive microbiology. J. Food Protect. **73(5)**:938-951.
8. Boons K, Van Derlinden E, Mertens L, Peeters V, Van Impe JF. 2013a. Effect of immobilization and salt concentration on the growth dynamics of *Escherichia coli* K12 and *Salmonella* Typhimurium. J. Food Sci. **78 (4)**:M567-M574.

9. Hills BP, Buff C, Wright KM, Sutcliffe LH, Ridge Y. 2001b. Microstructural effects on microbial survival: phase-separating dextran solutions. Int. J. Food Microbiol. **68**:187-197.
10. Brocklehurst TF , Mitchell GA, Smith AC. 1997. A model experimental gel surface for the growth of bacteria on foods. Food Microbiol. **14**:303–311.
11. Wright KM, Coleman HP, Mackie AR, Parker ML, Brocklehurst TF, Wilson DR, Hills BP. 2000. Determination of mean growth parameters of bacterial colonies immobilized in gelatin gel using a laser gel-cassette scanner. Int. J. Food Microbiol. **57**:75-89.
12. Theys TE, Geeraerd AH, Verhulst A, Poot K, Van Bree I, Devlieghere F, Moldenaers P, Wilson D, Brocklehurst TF, Van Impe JF. 2008. Effect of pH, water activity and gel micro-structure, including oxygen profiles and rheological characterization, on the growth kinetics of *Salmonella* Typhimurium. Int. J. Food Microbiol. **128**:67-77.
13. Meldrum RJ, Brocklehurst TF, Wilson DR, Wilson PDG. 2003. The effects of cell immobilization, pH and sucrose on the growth of *Listeria monocytogenes* Scott A at 10°C. Food Microbiol. **20**:97-103.
14. Lebert I, Dussap CG and Lebert A. 2004. Effect of a_w, controlled by the addition of solutes or by water content, on the growth of *Listeria innocua* in broth and in a gelatin model. Int. J. Food Microbiol. **94**:67-78.
15. Antwi M, Geeraerd AH, Vereecken KM, Jenné R, Bernaerts K, Van Impe JF. 2006. Influence of a gel microstructure as modified by gelatin concentration on *Listeria innocua* growth. Innov. Food Sci. Emerg. **7**:124-131.
16. Knudsen GM, Nielsen M-B, Grassby T, Danino-Appleton V, Thomsen LE, Colquhoun IJ, Brocklehurst TF, Olsen JE, Hinton JCD. 2012. A third mode of surface-associated

503 growth: immobilization of *Salmonella enterica* serovar Typhimurium modulates the
504 RpoS-directed transcriptional programme. Environ. Microbiol. **14(8)**:1855–1875.
505 17. Karina P, Julio C, Leda G, Noemi Z. 2011. Behavior of *Listeria monocytogenes* type1
506 355/98 (85) in meat emulsions as affected by temperature, pH, water activity, fat and
507 microbial preservatives.
508 18. Pivnick H, Engelhard WE, Thompson TL. 1954. The growth of pathogenic bacteria in
509 soluble oil emulsions. Papers in Microbiology. Paper 8.
510 19. Jarvis WR and Highsmith AK. 1984. Bacterial growth and endotoxin production in lipid
511 emulsion. J. Clin. Microbiol. **19(1)**:17-20.
512 20. Gilbert MRPh, Gallagherm SMS, Eads MMD, Elmore MMD. 1986. Microbial growth
513 patterns in a Total Parenteral Nutrition Formulation containing lipid emulsion. J.Pen.-
514 Parenter. Enter. **10(5)**:494-497.
515 21. Brocklehurst TF, Parker ML, Gunning PA, Coleman HP, Robins MM. 1995. Growth of
516 food-borne pathogenic bacteria in oil-in-water emulsions: II--Effect of emulsion structure
517 on growth parameters and form of growth. J. Appl. Bacteriol.**78**:609-615.
518 22. Hills BP, Manning CE, Ridge Y, Brocklehurst T. 1997. Water availability and the
519 survival of *Salmonella typhimurium* in porous systems. Int. J. Food Microbiol. **36**:187-
520 198.
521 23. Hills BP, Arnould L., Bossu C, Ridge Y. 2001a. Microstructural factors controlling the
522 survival of food-borne pathogens in porous media. Int. J. Food Microbiol. **66**:163-173.
523 24. Verrips CT, Smid D, Kerkhof A. 1980. The intrinsic microbial stability of water-in-oil
524 emulsions. II. Experimental. Eur. J. Appl. Microbiol. Biotech. **10**:73-85.

525 25. McKellar RC, Moir R, Kalab M. 2002. Factors influencing the survival and growth
526 of *Listeria monocytogenes* on the surface of Canadian retail wieners. J. Food Protect.
527 **57(5)**:387-392.
528 26. Francois K, Devlieghere F, Uyttendaele M, Standaert AR, Geeraerd AH, Nadal P, Van
529 Impe JF, Debevere J. 2006. Single cell variability of *L. monocytogenes* grown on liver
530 pâté and cooked ham at 7°C: comparing challenge test data to predictive simulations. J.
531 Appl. Microbiol. **100**:800-812.
532 27. Cayré EM, Vignolo G, Garro O. 2003. Modeling lactic acid bacteria growth in vacuum-
533 packaged cooked meat emulsions stored at three temperatures. Food Microbiol. **20**:561-
534 566.
535 28. Wimpenny JWT, Leistner L, Thomas LV, Mitchell AJ, Katsaras K, Peetz P. 1995.
536 Submerged bacterial colonies within food and model systems: their growth, distribution
537 and interactions. Int. J. Food Microbiol. **28**:299-315.
538 29. Farber JM, McKellar RC, Ross WH. 1995. Modelling the effects of various parameters on
539 the growth of *Listeria monocytogenes* on liver pâté. Food Microbiol. **12**:447-453.
540 30. Hwang C-A, Tamplin ML. 2005. The influence of mayonnaise pH and storage
541 temperature on the growth of *Listeria monocytogenes* in seafood salad. Int. J. Food
542 Microbiol. **102**:277-285.
543 31. Raghubeer EV, Ke JS, Campbell ML, Meyer RS. 1995 Fate of *Escherichia coli* O157:H7
544 and other coliforms in commercial mayonnaise and refrigerated salad dressing. J. Food
545 Protect. **58**:13-18.
546 32. Parker ML, Gunning PA, Macedo AC, Malcata FX, Brocklehurst TF. 1998. The
547 microstructure and distribution of micro-organisms within mature Serra cheese. J. Appl.
548 Microbiol. **84**:523-530.

549 33. Norton IT, Frith WJ. 2001. Microstructure design in mixed biopolymer composites. Food
550 Hydrocolloid. **15**:543-553.
551 34. Grinberg VYa and Tolstoguzov VB. 1997. Thermodynamic incompatibility of proteins
552 and polysaccharides in solutions. Food Hydrocolloid. **11(2)**:145-158.
553 35. Butler MF and Heppenstall-Butler M. 2003. Phase separation in gelatin/dextran and
554 gelatin/maltodextrin mixtures. Food Hydrocolloid. **17**:815-830.
555 36. Tromp RH, van de Velde F, van Riel J, Paques M. 2001. Confocal scanning light
556 microscopy (CSLM) on mixtures of gelatin and polysaccharides. Food Res. Int. **34**:931–
557 938.
558 37. Boons K, Mertens L, Van Derlinden E, David CC, Hofkens J, Van Impe JF. 2013b.
559 Behavior of *Escherichia coli* in a heterogeneous gelatin-dextran mixture. Appl. Environ.
560 Microb. **79 (9)**:3126-3128.
561 38. Baranyi J, Roberts T. 1994. A dynamic approach to predicting bacterial growth in food.
562 Int. J. Food Microbiol. **23**:277-294.
563 39. Sigma-Aldrich, Product Information Sheet Gelatin, retrieved from:
564 http://www.sigmaaldrich.com/content/dam/sigma-
565 aldrich/docs/Sigma/Product_Information_ Sheet/ 2/g9382pis.pdf at 22/07/2013.
566 40. Mattiasson B. 1983. Applications of aqueous two-phase systems in biotechnology. Trends
567 Biotechnol. **1(1)**:16-20.
568 41. Leja K, Dembczyński R, Białas W, Jankowski T. 2009. Production of dry *Lactobacillus*
569 *Rhamnosus* GG preparations by spray drying and lyophilization in aqueous two-phase
570 systems. Technol. Aliment. **8(4)**:39-49.

571 42. Umakoshi H, Kuboi R, Komasawa I. 1997. Control of partitioning of bacterial cells and
572 characterization of their surface properties in aqueous two-phase systems. J. Ferment.
573 Bioeng. **84(6)**:572-578.
574 43. Yagushi T, Dwidar M, Byun CK, Leung B, Lee S, Cho Y-K, Mitchell RJ, Takayama S.
575 2012. Aqueous two-phase system - Derived biofilms for bacterial interaction studies.
576 Biomacromolecules **13**:2655-2661.
577 44. Millqvist-Fureby A, Malmsten M and Bergenståhl B. 2000. An aqueous polymer two-
578 phase system as carrier in the spray-drying of biological material. J. Colloid Interf. Sci.
579 **225**:54-61.
580 45. Léonard L, Gharsallaoui A, Ouaali F, Degraeve P, Waché Y, Saurel R, Oulahal N. 2013.
581 Preferential localization of *Lactococcus lactic* cells entrapped in a caseinate/alginate
582 phase separated system. Colloid. Surface. B. **109**:266-272.
583 46. Harden VP, Harris JO.1953. The isoelectric point of bacterial cells. J. Bacteriol.
584 **65(2)**:198-202.
585 47. Schwarz –Linek J, Dorken G, Winkler A, Wilson LG, Pham NT, French CE, Schilling T,
586 Poon WCK. 2010. Polymer-induced phase separation in suspensions of bacteria. EPL.
587 89:68003p1-68003p6.
588 48. Dickson JS, Koohmaraie M. 1989. Cell surface charge characteristics and their
589 relationship to bacterial attachment to meat surfaces. Appl. Environ. Microb. **55(4)**:832-
590 836.
591 49. Kito M, Aibara S, Kato M, Ishinaga M, Hata T. 1973. Effects of changes in fatty acid
592 composition of phospholipid species on the β-galactoside transport system of *Escherichia*
593 *coli* K-12. Biochim. Biophys. Acta. **298**:69-74.

50. Zhang X-S, García-Contreras R, Wood TK. 2007. YcfR(BhsA) influences *Escherichia coli* biofilm formation through stress response and surface hydrophobicity. J Bacteriol. **189(8)**:3051-3062.
51. Sun G, Shen Y-I, Ho CC, Kusuma S, Gerecht S. 2009. Functional groups affect physical and biological properties of dextran-based hydrogels. J Biomed Mater Res A. **93A(3)**:1080-1090.
52. Schrieber R and Gareis H. 2007. Gelatin Handbook: Theory and Industrial Practice. WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim. Chapter 2, p59.
53. Gal S. 1974. Solvent versus non-solvent water in casein-sodium chloride water systems. *In* Duckworth R (ed), Water relations of foods, Academic Press, London.
54. Theys TE, Geeraerd AH, Devlieghere F, Van Impe JF. 2010. On the selection of relevant environmental factors to predict microbial dynamics in solidified media. Food Microbiol. **27**:220-228.
55. McClements DJ. 2006. Non-covalent interactions between proteins and polysaccharides. Biotechnol. Adv. **24**:621-625.
56. Koutsoumanis KP and Lianou A. 2013. Stochasticity in colonial growth dynamics of individual bacterial cells. Appl. Environ. Microb. **79(7)**:2294-2301.
57. Samapundo S, Ampofo-Asiama J, Anthierens T, Xhaferi R, Van Bree I, Szczepaniak S, Goemaere O, Steen L, Dhooge M, Paelinck H, Dewettinck K, Devlieghere F. 2010. Influence of NaCl reduction and replacement on the growth of *Lactobacillus sakei* in broth, cooked ham and white sauce. Int. J. Food Microbiol. **143**:9–16.
58. Robins MM, Wilson PDG. 1994. Food structure and microbial growth. Trends Food Sci Tech. **5**:289-29.

Figures

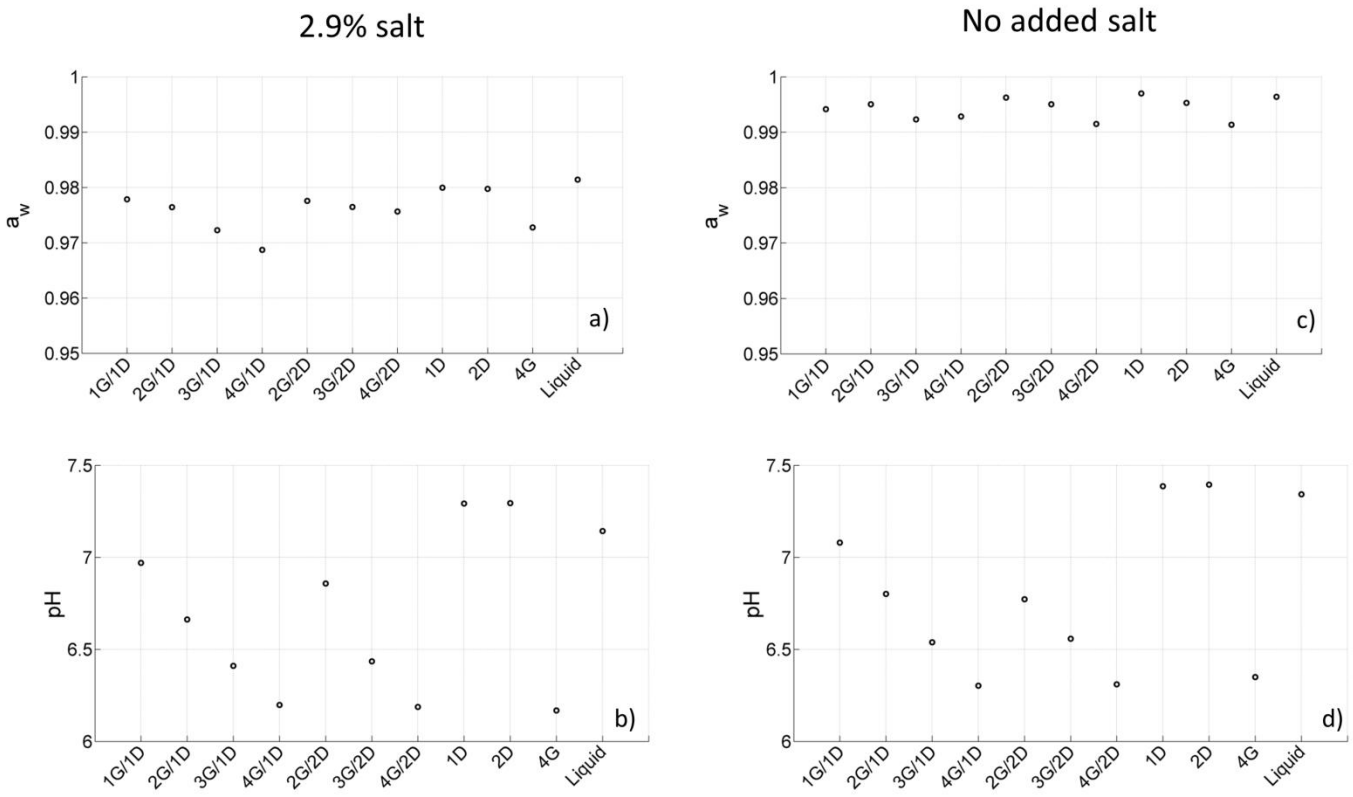
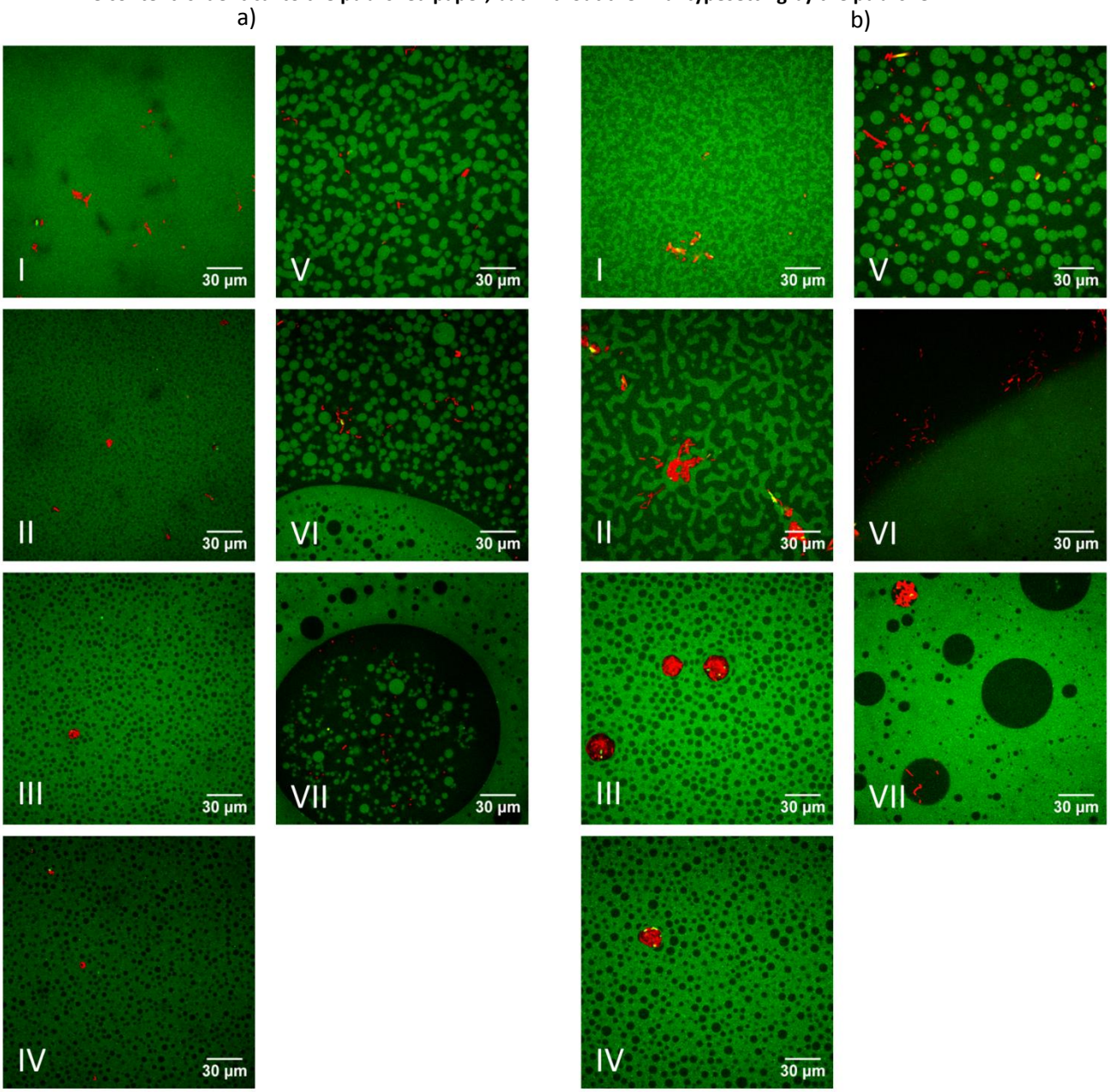
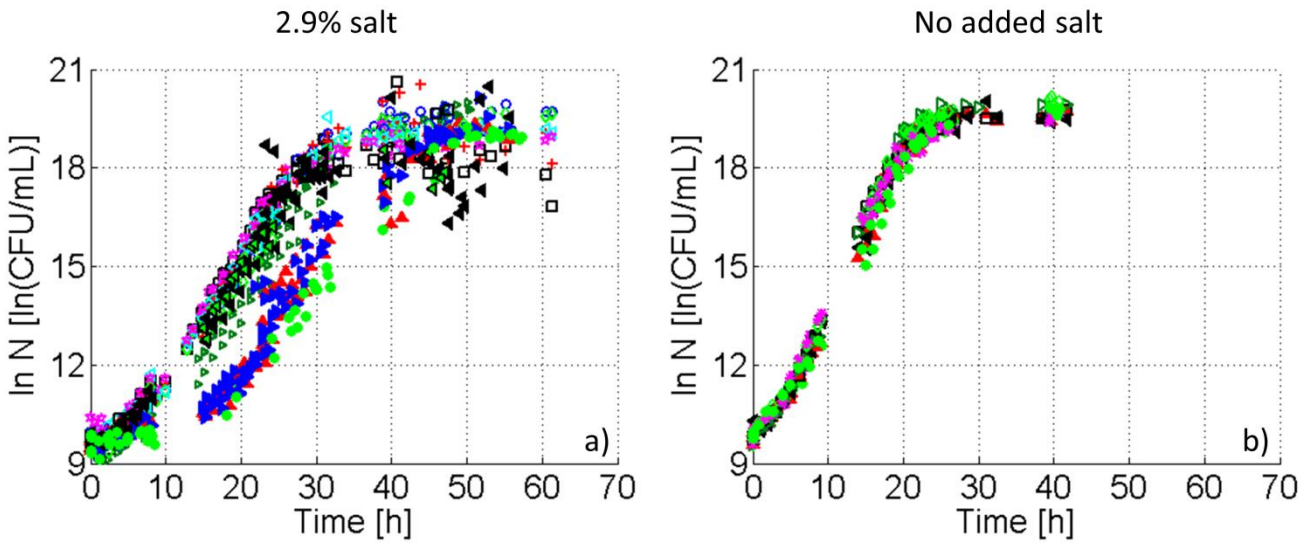


Figure 1: Water activity (a, c) and pH values (b, d) for the different binary and singular systems with (a, b) and without (c, d) salt.



621

622 Figure 2: Confocal microscope images of growth of *E.coli* JM-109 DE3 (red) in gelatin (G,
623 green)-dextran (D, black) mixtures with (a) and without added salt (b): I) 1G/1D, II) 2G/1D, III)
624 3G/1D, IV) 4G/1D, V) 2G/2D, VI) 3G/2D and VII) 4G/2D.



625

626 Figure 3: Population growth dynamics of *E. coli* JM-109 DE3 at 23.5°C in different mixtures of
627 gelatin (G) and dextran (D) with (a) and without salt (b): 1G/1D (>), 2G/1D (o), 3G/1D (+),
628 4G/1D (□), 2G/2D (◊), 3G/2D (<), 4G/2D (*), 1D (▲), 2D (►) , 4G (◄) and liquid (●).

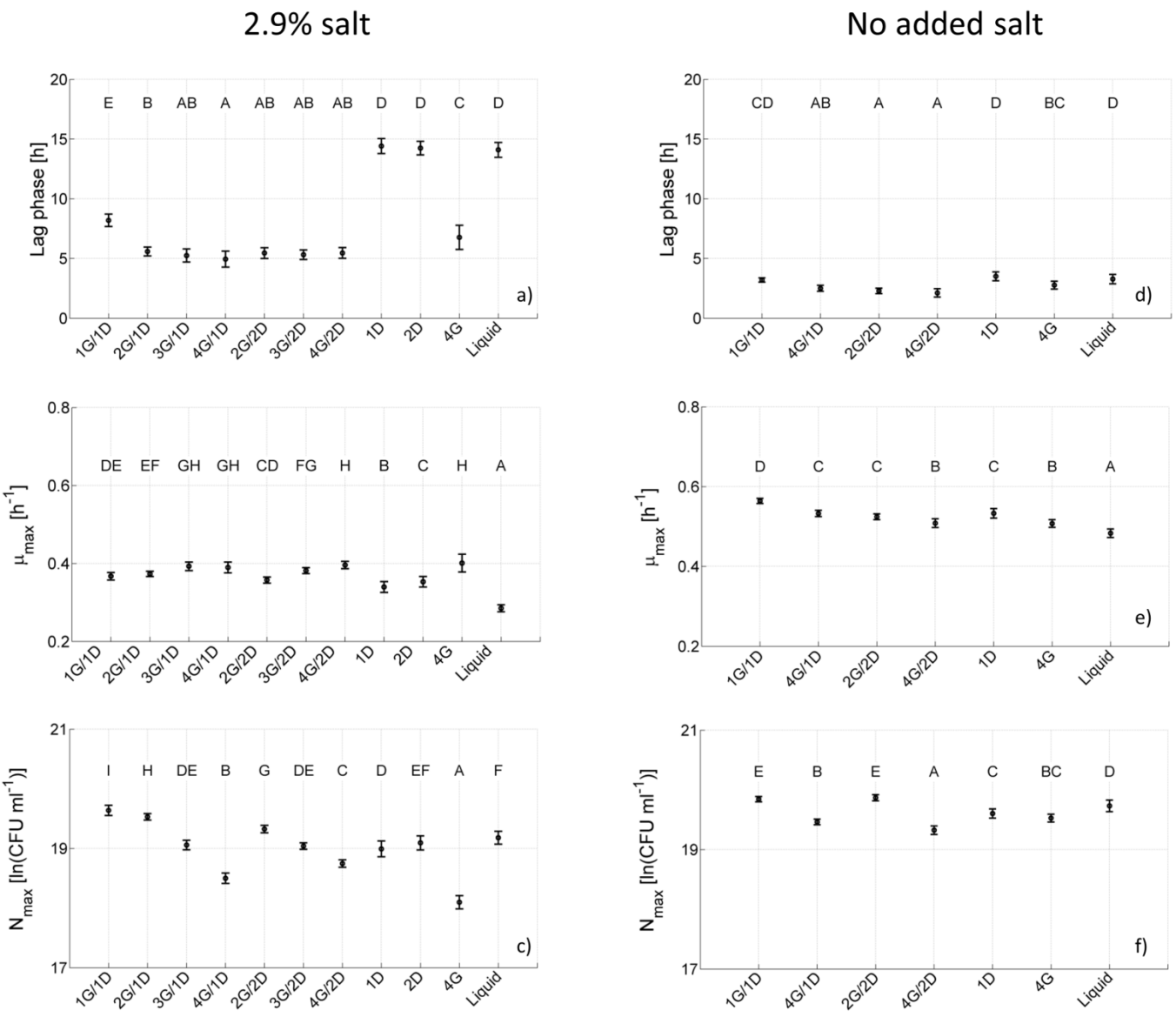


Figure 4: Population growth parameters and error bars for growth of *E. coli* JM-109 DE3 at 23.5°C in G/D binary and singular systems and BHI broth with (a, b, c) and without added salt (d, e, f): the duration of the population lag phase (a,d), the population maximum specific growth rate (b, e) and the population maximum cell density (c, f).

634 # Tables

635 Table 1: Overview of studies performed with different microorganisms, grown in homogeneous
636 and heterogeneous model systems and real food products.

		System	Microorganisms
Model systems	**Homogeneous systems**	Agar	*Salmonella* Typhimurium, *Listeria monocytogenes*, *E. coli* and other (5), *E. coli* (6)
		Carrageenan	*L. innocua* (7), *S.* Typhimurium and *E. coli* (8)
		Dextran	*E. coli* and *S.* Typhimurium (9)
		Gelatin	*S.* Typhimurium (10, 11, 12, 8), *L. monocytogenes* (13), *L. innocua* (14, 15)
		Pluronic F127-gel	*S.* Typhimurium (16)
		Xanthan gum	*S.* Typhimurium and *E. coli* (8)
	Heterogeneous systems	Meat emulsion	*L. monocytogenes* (17)
		Oil-in-water emulsion	*Salmonella* spp. (18), *Shigella* Sonnei and Dysenteria (18), *Klebsiella* Pneumoniae (18, 19), *Enterobacter cloacae* (19), *Staphylococcus aureus* (20), *E. coli* (20), *Pseudomonas aeruginosa* (20), *Canidida albicans* (20), *L. monocytogenes* (21), Yersinia enterocolitica (21)
		Packed bed of micro porous silica particles and Sephadex microspheres	*S.* Typhimurium (22, 23), *E. coli K12* (23), *Pseudomonas putida* (23))
		Water-in-oil-emulsion	*Citrobacter freundii* and *Kluyveromyces lactic* (24)
Real Food Products		Canadian retail Wieners	*L. monocytogenes* (25)
		Cooked ham	*L. monocytogenes* (26)
		Cooked meat emulsions	Lactic acid bacteria (27)
		Fermented sausages	Lactobacillus (28)
		Liver pâté	*L. monocytogenes* (29, 26)
		Mayonnaise	*L. monocytogenes* (30, 31)
		Serra cheese	Host micro flora (32)

637

638 Table 2: Percentages of gelatin and dextran mixtures used in the experiments.

	Gelatin (G) (% (w/v))	Dextran (D) (% (w/v))
1G/1D	2.5	2.5
2G/1D	5.0	2.5
3G/1D	7.5	2.5
4G/1D	10.0	2.5
2G/2D	5.0	5.0
3G/2D	7.5	5.0
4G/2D	10.0	5.0
1D	0.0	2.5
2D	0.0	5.0
4G	10.0	0.0
Liquid	0.0	0.0

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640